ANTIMICROBIAL RESISTANCE PROFILES AND CLONAL RELATEDNESS OF *PSEUDOMONAS AERUGINOSA* STRAINS RECOVERED FROM WOUND INFECTIONS OF PATIENTS PRESENTING IN TIGONI DISTRICT HOSPITAL, KENYA

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Antimicrobial Resistance Profiles and Clonal Relatedness of *Pseudomonas aeruginosa* Strains Recovered from Wounds Infections of Patients Presenting in Tigoni District Hospital, Kenya

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DECLARATION

This Thesis is my original work and has been not been submitted to any University for award of a Degree.

Signature……..Date…….

Thomas Gachuki Thuo

This Thesis has been submitted for examination with our approval as University Supervisors

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DEDICATION

I dedicate this work to my wife and children for the support during times I worked on this thesis.

AKNOWLEDGEMENT

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LIST OF ACRONYMS AND SYMBOLS

µg-	Microgram
µl -	Microlitre
ml-	Millilitre
mg-	Milligram
mm-	Millimeter
AME-	Aminoglycoside Modifying Enzyme
ATCC-	American Type Culture Collection
BASC-	British Standard for Sensitivity committee
bla **-**	β-lactamase
CLSI	clinical laboratory standards institute
ESBL –	Extended spectrum B- Lactamases
ITROMID-	Institute of Tropical Medicine and Infectious Diseases
JKUAT-	Jomo Kenyatta University of Agriculture and Technology
KEMRI-	Kenya Medical Research Institute
MBL -	Metallo- B- Lactamase
MH-	Mueller-Hinton agar
MIC-	Minimum Inhibitory Concentration

***bla*$_{\text{NDM-1}}$-**	New Delhi Metallo- B- Lactamase
NPHLS-	National Public Health Laboratories
NMRL-	National Microbiology Reference Laboratory
PCR-	Polymerase Chain reaction
RPM-	Revolutions per Minute
SPSS-	Statistical Package for the Social Sciences
SBA	Sheep Blood Agar
***bla*$_{\text{SHV}}$-**	Sulfhydryl variable β--lactamase
***bla*$_{\text{TEM}}$-**	Temoneira
UTIs-	urinary tract infections
UVL-	Ultra Violet Light

DEFINITION OF TERMS

GTG_5 fingerprinting: This low-resolution method detects small tandem repeats in bacteria DNA and generates polymorphic patterns essential in distinguishing bacterial strains. Bacterial strains with more similar tandem repeats tend to be closely related and may infer clonal expansion in a particular ecosystem.

ABSTRACT

Pseudomonas aeruginosa is a leading cause of hospital infections and is intrinsically resistant to most antimicrobials. Emergence of multidrug resistant *Pseudomonas aeruginosa* has been reported and poses a great challenge in the management of the resultant infections. While a sizable amount of research has been done on these infections in developed countries, little is known about the susceptibility profiles and molecular diversity of strains recovered from wounds in Kenya. This cross-sectional study conducted in Tigoni District Hospital in rural Kenya sought to determine susceptibility profiles, molecular diversity of strains and risk factors associated with carriage of the organism in wound infections. This being a rural area, residents are presumed to be more likely to get wounds due to the manual nature of their work. Wound swabs were collected from 299 patients, and transported to the NMRL. Isolation was done on SBA and MacConkey with salt then drug susceptibility testing for *Pseudomonas aeruginosa* isolates using gentamicin, amikacin, ciprofloxacin ,piperacillin-tazobactam, ceftazidine, cefepime and meropenem. PCR was performed testing for β lactamase genes *bla* $_{\text{NDM-1}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{TEM.}}$ Fingerprinting analysis was done using the $(GTG)^5$ primer to determine the phylogeny of recovered isolates. Statistical analysis of age and sex in relation to *Pseudomonas aeruginosa* carriage and recorded antimicrobial resistance patterns was done using IBM SPSS version 20 software (SPSS, Inc. Chicago, IL). Chi-square test was used to calculate P-value for risk factors associated carriage of *Pseudomonas aeruginosa* in wound infections. Binary logistic regression analysis was done to generate the adjusted odds ratio with 95% confidence interval, an alpha of less than 0.05 ($P < 0.05$) was considered statistically significant. Of the 299 patients 85 (28%) had positive wound carriage of *Pseudomonas aeruginosa*. This was highest among adults (94%) compared to children (6%)(P: 0.001, C.I:2.1-14.0, O.R:3.4). Wounds in females were more likely to be colonised (67%) compared to those in males at (33%) (P: 0.19, C.I:0.84-2.4, O.R: 1.42). Patients sourcing medication from a community chemist were more likely to have bacteria carriage compared to those who sourced from a hospital pharmacy (P: 0.001, C.1:3.01-8.86, O.R:5.17)**.** Those who acquired antimicrobials without a doctor's prescription were more likely to be colonized compared to those to those had (P: 0.001, C.I:3.01-8.86, O.R:5.17). Patients who did not complete dosage had a higher carriage (50.6%) compared to those that completed (49.4%). Highest antimicrobial resistance was recorded towards Ceftazidime (64%), Cefepime (52%) while Piperacillin-tazobactam was least resisted (20%). The isolates were more resistant towards Gentamicin (45%) and Amikacin (40%) compared to Ciprofloxacin (25%). Notable is the high resistance towards Meropenem (40%). Carriage of *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{NDM}}$ was most common among strains showing resistance towards third generation cephalosporins, in addition to Amikacin and Meropenem for *bla* $_{\text{NDM-1}}$Tight clustering was noted in isolates from diverse patients with a similarity of $\geq$ 80% was noted in 9 clusters based on isolates banding patterns. This appearance of *bla*$_{\text{NDM-1}}$ linked to pervasive misuse of carbapenem is worrying. Strengthening antimicrobial stewardship is recommended

CHAPTER ONE

INTRODUCTION

1.1 Background information

The β-lactams antimicrobials, such as cephalosporin and carbapenems, aminoglycosides and fluoroquinolones are usually the drugs of choice for the treatment of Pseudomonal infections. The production of extended spectrum β-lactamases for example NDM-1 SHV and TEM offers a mechanism of resistance to the wide range of antimicrobials that contain the β-lactam ring.

There has been reported increased resistance to carbapenems in the developed world; however information is limited in developing countries. There are few reported cases of Metallo β-lactamases such as NDM-1 in Africa, which confers resistance to carbapenems (Kilivwa *et al.*, 2018; Pitout *et al*., 2008; Ssekatawa *et al.*, Ejobi *et al*., 2018). Worse still there is no information on these resistance genes in wounds infections from rural areas in Sub-Sahara Africa where over 60% of people live (WHO 2011, 2011). Rural areas are typified by low income, increased hazards that would cause wounds, poor laboratory infrastructure, hospitals lacking essential antimicrobials and an emerging increasingly empowered population that will readily resort to self-medication. Development of resistance is linked to improper use of antimicrobials and the transmission of resistance genes (Weist & Hogberg, 2016).

This study sought to determine the prevalence of wound infections caused by *Pseudomonas aeruginosa* in Tigoni in rural Kenya, antimicrobial susceptibility patterns and presence of carbapenemases and extended spectrum β-lactamases resistance genes. The likelihood for getting wounds in Tigoni is high due to the largely manual work by the people living in the area. This is either manual agricultural or informal/formal industrial work with inadequate protection. The area is also characterised by low income which impacts negatively on patient's access to proper care for wounds in hospitals and

ability to afford proper antimicrobials. The study sought to establish some of the risk factors that may be associated with wound carriage of *Pseudomonas aeruginosa* and resistant strains. A questionnaire was used for capturing social demographic features of the study participants.

1.2 Statement of the problem

Pseudomonas aeruginosa remains one of the common causes of wound infections that have widely been documented (Peleg & Hooper, 2010). While most of these infections have been documented among surgical and burn wounds patients elsewhere (Decraene *et al.,* 2018; Egbe *et al*., 2011), very little has been done to determine the prevalence of this bacteria strain in traumatic and non-traumatic wounds in Kenya. Information on risk factors that possibly leads to both traumatic and non-traumatic wound colonization by *Pseudomonas aeruginosa* has not been documented in Kenya. This study was conducted in rural Kenya among farming community which make this a unique study. Farmers are likely to have more injuries than typical urban population due to mechanical nature of their occupation, however, this hypothesis has not been tested either. Although the study did not take samples from the environment, it is important to note that *Pseudomonas aeruginosa* is ubiquitous on un-animate objects which may be a source of bacteria colonization. There is also a research gap in determining the genetic relatedness of *Pseudomonas aeruginosa* that colonize wound infections especially within a community.

While abundant data on antimicrobial resistance is available on *Pseudomonas aeruginosa* from surgical and burn wounds are available *(Decraene et al., 2018; Egbe et al.,* 2011), no data is available on the same from strains from non-traumatic wounds of patients in a rural set up. The ease in access and affordability of antimicrobial agents especially among the urban population has been termed as one of many factors that leads to antimicrobial resistance emergence. The emergence and spread of in genes encoding for β-lactamases which mediates resistance to carbapenems have further limited treatment options for infections associated with *Pseudomonas aeruginosa*. The development of resistance has been documented to significantly increase the risk of

fatality in some cases up to three times have increased mortality risk as a result of treatment failure and prolonged hospitalization. It is however worth noting the paucity of data available on antimicrobial sensitivity pattern of bacterial strains recorded from rural inhabitants in Kenya where accessibility and affordability to these agents is comparatively limited compared to the urban population.

Pseudomonas aeruginosa has also been shown to lack gene duplication, which is an indicator that the size of its genome is due to greater gene and functional diversity (Silby *et al*., 2011). The multidrug efflux systems are members of the resistance-nodulation-cell division (RND) family. Each of the genes encoding a putative RND transport protein is adjacent to a gene for a probable membrane fusion protein; most RND loci also contain genes for outer membrane proteins of the OprM family (Stover et al., 2000). A common efflux pump MexAB-OprM, is made of a pump (MexB), a linker lipoprotein (MexA), and an exit portal (OprM). Although the study will not screen for these genes that can also been attributed to antimicrobial resistance, there is need for future studies check possible carriage in *Pseudomonas aeruginosa* isolates in Kenya where this has not been documented. Saaid *et al.,* 2016 Farshadzadeh *et al.,* 2014

1.3 Justification

Since *Pseudomonas aeruginosa* is among the most common cause of wound infections, it is important to determine possible risk factors that may cause wound colonization by this bacteria strain. Such information is particularly essential in formulation of mitigation measures by public health officials. There is therefore dire need for basic surveillance to provide research based data on possible risk factors especially among rural central Kenya where no studies have been conducted. *Pseudomonas aeruginosa* is one of the most common bacterial strains that have been associated with high intrinsic resistance to multiple antimicrobial agents (Cicek *et al.,* 2014). Infections caused by *Pseudomonas aeruginosa* are therefore becoming increasingly difficult to manage due to emergence and spread of antimicrobial resistance. The implication of such resistances has been prolonged hospitalization and delayed healing of wounds. Regular monitoring the

effectiveness of antipseudomonal agents through surveillance is essential in informing the best treatment of infections associated with *Pseudomonas aeruginosa* In this cross sectional research study, we intended to provide missing data of antimicrobial resistance patterns, genetic basis of resistance and strains relatedness among a farming community.

1.4 Research questions

1. What is the prevalence of *Pseudomonas aeruginosa* in wound infections among patients attending Tigoni district hospital?
2. What are the antimicrobial resistance patterns to anti-pseudomonal agents in isolates recoverable from these wounds?
3. What are the possible risk factors for carriage of multi-drug resistance *Pseudomonas aeruginosa* strains in infected wounds?
4. What proportion of *Pseudomonas aeruginosa* isolates carry most common carbapenemases and extended β-lactamases genes.
5. Are the recovered *Pseudomonas aeruginosa* isolates genotypically related?

1.5 Research objectives

1.4.1 Main objective

To determine antimicrobial resistance profiles and clonal relatedness of *Pseudomonas aeruginosa* strains recovered from wounds infections of patients presenting in a rural hospital in Kenya.

1.4.2 Specific objectives

1. To determine the prevalence of *Pseudomonas aeruginosa* in wound infections among patients attending Tigoni District Hospital?
2. To determine antimicrobial resistance patterns of *Pseudomonas aeruginosa* recovered from wound infections

3. To determine the risk factors associated with carriage of *Pseudomonas aeruginosa* in wound infections?
4. To determine the carriage of Extended Spectrum β lactamases and carbapenemases in recovered *Pseudomonas aeruginosa* isolates
5. To determine genetic relatedness of *Pseudomonas aeruginosa* wound isolates.

CHAPTER TWO

LITERATURE REVIEW

2.1 Epidemiology of wound infections

Pseudomonas aeruginosa has a variety of virulence factors that contribute to its pathogenicity which include production of exotoxins, proteolytic enzymes and hemolysins that destroy cells and tissue. Some strains produce alginate, a polysaccharide polymer that inhibits phagocytosis. (Maharjan *et al.,* 2018)Nevertheless, the organism remains an opportunistic pathogen that requires a compromised host to establish an infection. The organism either disrupts or takes advantage of loss of protection offered by intact epidermis in cases like of burns, puncture wounds, eye trauma. Other infections include infections of the respiratory tract (hospital acquired pneumonia in patients in respirators), urinary tract, wounds, blood, central nervous system, burns, ear and cystic fibrosis (Rouhi & Ramazanzadeh, 2018). The organism has been incriminated as a leading cause of wound infections (Siddiqui & Bernstein, 2010 Agyepong *et.al.,* 2018, Tarana & Shamsuzzaman, 2018 2015, Pathi *et al.,* 2013. Satyajeet *et al.,* 2014 Mohan *et al.,* 2013). A wound is a break in the skin which can either be acute or chronic. An acute wound is caused by damage to the skin which can be accidental or surgical. A chronic wound is one that does not heal in the normal manner and duration mostly due to underlying pathological cause for example diabetic wounds or bed sores. Whereas both types of wounds are prone to infections when a wound does not heal the likelihood of developing an infection is increased. This is when the organism's virulence factors overcome the host defence mechanisms. The formation of biofilms has also been shown to play a huge role in *Pseudomonas aeruginosa* wound infections (Maharjan, *et al*., 2018). In compromised patients such infections are often severe and often life threatening. These infections have high mortality which is associated with ineffective empirical therapy (Cicek *et al.,* 2014; Siddiqui & Bernstein, 2010).

Patients with infected non-healing wounds pose a huge problem for hospitals at times taking up to 50% of medical ward beds and strain already overstretched facilities They often lead to prolonged duration of stays in hospitals which drives up costs gobbling up to 4% of health care budgets and cause high mortality of 7.6% (Nathwani *et al.,* 2014, Finn Gottrup *et al.,* 2014, Turner KH *et al.,* 2014).The effective treatment of these wound and skin infections is hampered in a great way by presence of multiple drug resistant bacteria (Oli *et al*., 2017). The development of resistance statistically significantly increases the risk of fatality in some cases up to three times have increased mortality risk. These same patients also have a longer length of hospital stay. (Nathwani *et al.,* 2014, Hong-Tu *et al*., 2015).

In a study at military burn centre in the USA *Pseudomonas aeruginosa* was found to be the most frequent cause of burn-wound infections. (Keen *et al.,* 2010). It was the most common bacteria causing wound infections. (Raffaele *et.al*., 2015). In a Pakistani study on chronic wounds the organism was the most prevalent organism at 27% (Rahimm *et al.,* 2016). In neighboring India the organism was the most commonly isolated from pus and wound swabs. (Tarana & Shamsuzzaman, 2018). More than 80% of the *Pseudomonas aeruginosa* isolates were obtained from wound and pus swabs with, 20.69% of these being multidrug resistant in another Indian study (Chander *et al*., 2013). *Pseudomonas aeruginosa* was the leading cause of wound infections and pus swab isolate in a Ghanaian study. (Agyepong, *et al*., 2018).Yet in another Ghana study the organism recorded the highest prevalence of 30.7% in wound infections (Amankwa *et al.,* 2017). In two Nigerian studies on wound infections the organism was the leading causative bacteria as an ESBL producer at 34.92% and 36.07% (Angus *et al.,* 2017,).In an Egyptian study multidrug ESBL producing were isolated from burn wounds 57% and pus swabs 66%. (Mahmoud *et al.,* 2013). In Sub Sahara Africa a study on wound infections at the Mhumbili hospital in Tanzania, the most prevalent bacterial species were *Pseudomonas aeruginosa* at 16.3% (Manyahi *et al*., 2014).

2.2 Resistance towards carbapenem

Resistance to carbapenems, which is often accompanied by resistance to multiple other agents, has increased. (Mushi *et al.*, 2014). A study in China found that over a 9 year period that there was a 4.6% increased resistance to meropenem which was the highest as compared to other drugs (Xu, *et al.*, 2013).Carbapenems have been shown to have limited activity of 4% sensitivity against ceftazidime resistant *Pseudomonas aeruginosa* in another China study (Jean, *et al*., 2002).In Europe the average drug resistance of *Pseudomonas aeruginosa* increased from 17.1% in 2012, to 17.8 % in 2015 with a reported resistance carbapenems of 52% and 66% in Slovakia and Romania respectively (European CDC surveillance report 2015*).* High levels of resistance to ceftazidime 73.7% and 76% meropenem were observed in study is US hospitals 8.9% of *Pseudomonas aeruginosa* were classified as multidrug-resistant (Farrell, *et al*., 2014). The organism was the cause of 75% of chronic wound samples of which 6% were multidrug resistant in a Brazilian setting (de Oliveira et al., 2017). Clinical isolates from burns wounds in Iran had 100% resistance to two carbapenems Meropenem and Imipenem (Akbarmirsalehian *et al.*, 2017). Isolates from burn wounds showed 78% multi drug resistance with 42% of this producing ESBL (El-Shouny *et al*., 2018). A study by Ahmed *et al* in Egypt found out that increased resistance to β-lactams and third generation cephalosporins of imipenem at 34%, cefepime 98%, piperacillin \ tazobactam at 94.7% and ceftazidime at 91% (Ahmed *et al*., 2009). A study in Nigeria reported 50% prevalence of *P, aeruginosa* from wound specimen where most of these isolates were also MDR(Pondei *et al*., 2013). In Ethiopia, the same organism was second most common in cause of wound infections with a multidrug resistance rate of 73 % (Mulu *et al*., 2017). In a Ugandan study the multidrug resistance was 81% with a 24% resistance to carbapenems(Kateete *et al.*, 2016). In a study in Tanzania the organism was the second most common cause at 18.2%, ESBL producing gram negative bacteria showed resistance rate 100% to ceftriaxone and cefotaxime (Kassam *et al*., 2017). Another Tanzanian study 73% of the 15 isolates with reduced susceptibility to meropenem were positive for carbapenemases genes (Mushi *et al.*, 2014).

The resistance gene *bla*$_{\text{NDM-1}}$ was first reported in Africa in Kenya in 2011 in *Klebsiella.* There has been reported incidence of NDM-1producing *Pseudomonas aeruginosa* in Uganda in 2016, and Egypt in 2014 (Kateete *et al*., 2016; Zafer *et al.*, 2014). A study at the Aga Khan hospital in Nairobi found the prevalence of Metallo-β-lactamase producing *Pseudomonas aeruginosa at* 13.7% (Pitout *et al.*, 2008).

Previous exposure to antimicrobials has been linked to development of resistance in *Pseudomonas aeruginosa* either to same or different antibiotic because it harbors multiple mechanisms of resistance (Shick, 1989)A key factor that plays a role in widespread dissemination of β-lactams and Carbapenem resistant bacteria is the over the counter misuse of antibiotics or excessive use of antibiotics which are either generic or counterfeits. The restricted use of imipenem has been shown to reduce rates of resistance. (Chander *et al.,* 2013).In this case as opposed to other antibiotics carbapenems are more expensive and therefore original drugs are not widely available in rural settings characterised by high poverty levels but rather generics or counterfeits. Other factors that lead to dissemination of carbapenem resistant bacteria are presence in hospital environments, weak infection control practises and lack of antimicrobial stewardship (Manenzhe *et al.*, 2015, Decraene et al. 2018;Kohlenberg *et al.*, 2010). There is lack of antimicrobial resistance data in Africa which eventuates in ineffective responses, poor antimicrobial stewardship and late detection of dissemination of resistant bacteria. (Manenzhe *et al.*, 2015).

2.3 Genetic complexity and resistance mechanisms

The organism possesses intrinsic resistance which is due to three factors; inactivating enzymes, low permeability of its cell wall to antimicrobials and active efflux pumps (Riou *et al.*, 2010). The complementary effect of these independent mechanisms offers the organism wide ranging resistance to most potent drugs. (WHO, 2008).

2.4 Molecular basis of resistance

Pseudomonas aeruginosa contains the highest proportion of regulatory genes observed for a bacterial genome. A large number of these genes are involved in the catabolism, transport and efflux of organic compounds as well as four potential chemotaxis systems. The size and complexity 5,570 predicted open reading frames (ORFs), of the genome acts as evolutionary adaptation permitting it to thrive in diverse environments and resist the effects of a variety of antimicrobial substances. The organism has nearly 300 cytoplasmic membrane transport systems, about two-thirds of which are involved in the import of nutrients and other molecules. These numerous cytoplasmic transport systems afford the organism a wide capacity to metabolize and grow on organic substances and are numerous iron-siderophore uptake systems. They enhance the ability to export compounds for example, enzymes and antimicrobials by a large number of protein secretion and resistance-nodulation-cell division efflux systems. The efflux systems are made of a protein transporter of the cytoplasmic membrane that uses energy in the form of proton motif force to transport drugs and other substances through the inner membrane, a periplasmic connective protein and an outer membrane protein component with a barrel configuration. A large number of genes codes for outer membrane proteins which play a role in cell surface exposure, transport of antimicrobials, export of extracellular virulence factors and assist in adhesion and motility by anchoring the structures involved. *Pseudomonas aeruginosa* has been shown to lack recent gene duplication, which is an indicator that the size of its genome is due to greater gene and functional diversity (Silby *et al.,* 2011) the multidrug efflux systems are members of the resistance-nodulation-cell division (RND) family. Each of the genes encoding a putative RND transport protein is adjacent to a gene for a probable membrane fusion protein; most RND loci also contain genes for outer membrane proteins of the OprM family (Stover *et al.*, 2000). A common efflux pump MexAB-OprM, is made of a pump (MexB), a linker lipoprotein (MexA), and an exit portal (OprM).

Pseudomonas aeruginosa may develop resistance to carbapenems through combined mechanisms such as target inaccessibility, stable derepression of AmpC b-lactamase,

overexpression of efflux systems and production of Metallo β-lactamases (MBLs) (Pournaras *et al.,* 2007).

Commonly, genetic information for target site or efflux resistance mechanisms is chromosomally encoded. There has however been recent emergence of plasmid-mediated transferable resistance.(Exner *et al.,* 2017). Transfer of resistance genes from species found in the environment, creates a problem in the treatment of these infections (Falagas *et al.,* 2005).

In Kenya various studies have documented carriage of bla_{VIM}, bla_{SHV} and bla_{NDM} in *Pseudomonas aeruginosa* isolates from clinical isolates consisting of blood, urine, tracheal aspirates and pus samples (Kilivwa *et al.,* 2018; Pitout *et al.,* 2008). To the best of my knowledge data on antimicrobial resistance patterns of *Pseudomonas aeruginosa* from wound infections and their resistance mechanisms have not been documented in Kenya.

2.5 Treatment options

Treatment of *Pseudomonas aeruginosa* infections involves the presumptive use of combination therapy while awaiting susceptibility results. Once susceptibility is determined a single drug is used. This is because *Pseudomonas aeruginosa* readily acquires resistance to the available antimicrobials.

Therapy usually involves the use of antimicrobials with anti-pseudomonal activity. These include anti-Pseudomonal penicilins such as Ticarcillin and piperacillin (with or without β-lactamase inhibitors such as Tazobactam), and third and fourth generation cephalosporins such as ceftazidime and cefepime. Others drugs include aminoglycosides for example amikacin, gentamicin, aztreonam, oxazolidinones, and carbapenems such as imipenem and meropenem. The list also includes fluoroquinolones such as ciprofloxacin and levofloxacin, colistin, and polymixin B (Kanj & Kanafani, 2011). It is recommended that patients with severe *Pseudomonas* multidrug resistance (MDR) infections should be

treated with a combination therapy consisting of an anti-pseudomonal β-lactam such as meropenem (carbapenems), an aminoglycoside for example amikacin or fluoroquinolones such as ciprofloxacin to provide adequate therapy cover and improve patient outcomes.

Antimicrobial resistance in *Pseudomonas aeruginosa* may easily emerge during treatment(Zavascki, *et al*., 2010). Resistance to aminoglycosides for example gentamicin and amikacin involves the MexXY-OprM efflux pump as well as the aminoglycosides modifying enzymes (AMEs). Resistance to fluoroquinolones for example ciprofloxacin involves mutations in target genes (*gyrA*, *gyrB*, *parC*, and *parE*), aminoglycoside modifying enzymes or to drug efflux systems (MexAB-OprM, MexCD-OprJ, MexAB-OprM, MexXY-OprM, OqxAB and Qep) (Breidenstein *et al*., 2011). The organism possesses intrinsic resistance to the majority of antimicrobial agents, and through the different mechanisms develops combined resistance to multiple antimicrobial groups (Mushi *et al.*, 2014).

2.6 Metallo β-lactamases

β-lactams are antimicrobials that contain the β-lactam ring in their molecular structure and include cephalosporins for example ceftazidime (third generation), cefepime (fourth generation) carbapenems for example meropenem, monobactams for example aztreonam and penicillin derivatives for example piperacillin. Some drugs are β-lactam inhibitors for example tazobactam and are used in combination with penicillin derivatives for treatment of *Pseudomonas aeruginosa* infections for example piperacillin. Resistance to carbapenems for example meropenem develops due to more than one mechanism with efflux pump systems playing a major role with a key role the mediation by Carbapenemases which include β-lactamases hydrolysing carbapenems among other β-lactams. The β-lactamases are classified in Ambler's structural classification into four molecular classes, two of which were the focus of this study the Extended Spectrum β-Lactamases and Metallo-β-Lactamases (Bush & Jacoby, 2010).

Derive from genes for narrow spectrum β-lactamases for example bla_{SHV} and bla_{TEM}. Genetic mutations alter the amino acid configuration on the active sites. They are found in plasmids that are easily transferred to different bacterial species. (Fuste *et al.*,2013)

The broad spectrum Metallo- β lactamases are thought to be most clinically significant for three reasons: genes encoding for them are found as gene cassettes in integrons, are transferable and worse still other resistance genes for other antibiotic classes can also be found in the same integrons resulting in multi-drug resistance. The bla_{NDM-1} a type of MBL can hydrolyse all β-lactam antimicrobials except for monobactams. (Fuste *et al*.,2013)

Carbapenems enter into the periplasmic space of *Pseudomonas aeruginosa* through the OprD outer membrane porin. Meropenem is stable to dehydropeptidase I inactivation and is more active against gram-negative bacteria and especially against *Pseudomonas aeruginosa* because it passes more swiftly through the OprD porin (Meletis *et al*., 2012).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

This study was conducted in Tigoni District Hospital, in rural Kiambu County, Kenya. Majority of residents here live in poverty (below 2 dollars a day) and therefore access to proper health-care is a major problem to many people hence opting for self-medication. Majority of residents rely on agriculture and other manual work for a livelihood which makes them prone to suffer wounds. This location typical of many rural areas in Kenya is unique as health facilities are not well equipped lacking necessary antimicrobials leading to a flourishing industry of private chemists.

3.2 Study design

A consecutive random sampling method was used in this cross-sectional study to obtain wound swabs from consenting patients attending Tigoni District hospital for a period of six months from June to December 2015.

3.3 Patients recruitment

Individuals having a wound infection were identified and recruited at the outpatient department. Identification of the in-patient population having wound infections was done by a nurse at the medical and surgical wards. Recruitment process involved brief introduction of the research study and potential benefits in involvement which includes proper diagnosis.

3.4 Inclusion criteria

i. To be eligible for recruitment one must have been a resident of Kiambu
ii. Only patients with a wound infection were recruited.
iii. One must be willing to participant in this study by signing a written consent

iv. This study only recruited minors who agreed to participate accompanied by a signed assent from the parent or guardian.

3.5 Exclusion criteria

i. Non-Kiambu residents were excluded from this study.
ii. Patients un willing to participate in this study by signing a written consent were also excluded

3.6 Sample size

The Fisher's *et al* 2005 method was used to calculate the sample size using a previously published prevalence of 14%. (Kasiulevičius *et al.*,2006)

$N = Z^2 P (l-P)/d^2$

Where N = Minimal sample size:

Z = Standard normal deviation corresponding to 95% confidence interval (=1.96);

P = Prevalence of 8% (Ngumi *et al.*, 2010)

d = degree of precision (5%)

- $N= 1.96^2 X 0.14(0.86)/0.05^2$

Therefore, a minimum of 246 wound samples were obtained from different patients seeking treatment at Tigoni District Hospital.

3.7 Sample collection and demographic data capture

In order to collect the specimen, a sterile cotton wool swab was gently rotated on the patients' wound. The swabs were transported in Cary-Blair media in a cool box below

10°C to the National Microbiology Reference Laboratory within three hours of collection. Unique codes were assigned to sample collection forms and transport media. A total of 299 non-duplicate wound specimens were collected and analyzed in this study. Of these specimens, 240 (80%) were from adults and 59 (20%) were from children. Further, 183 (61%) were obtained from males compared to 116 (39%) from females. The mean age was 33.6 years and median age 29 years.

Social demographic data was captured included patient's age, gender, residence, antimicrobial source and ease in accessing over the counter, prescription availability was captured using a structured questionnaire.

3.8 Bacteria isolation and identification

Pus and wound swabs were directly plated on sheep blood agar (SBA) and MacConkey without salt and incubated at 35^0 C, for up to 24 hours. On MacConkey agar plate, non-lactose fermenters colonies with characteristic dry translucent edges were presumed as *Pseudomonas aeruginosa*. On SBA, *Pseudomonas aeruginosa* colonies were shiny, dry with rough edges, β-hemolytic with grape like smell. Identification was done using Gram stain and a series of biochemical tests which includes; Oxidase (positive), glucose fermentation, hydrolysis of arginine, and nitrate production test among others as previously described(Al-Charrakh, *et al.,* 2016).

3.9 Antimicrobial susceptibility testing

Antimicrobial susceptibility tests were performed using Kirby–Bauer disc diffusion technique on Mueller Hinton agar (Oxoid). A suspension of the organism in normal saline equivalent to McFarland turbidity standard of 0.5 was evenly spread three times on Mueller Hinton plate to obtain a confluent lawn. Antimicrobial discs selected based on CLSI guidelines were included; gentamicin (GEN 10 µg), amikacin (AK 30µg), ciprofloxacin (CIP 5 µg), piperacillin-tazobactam (TZP 100/10 µg), ceftazidime (CAZ 30 µg), cefepime (FEP 30 µg) and meropenem (MEM 10 µg) were then dispensed.

Pseudomonas aeruginosa strains ATCC 27853 was used as control strain for media quality and disc potency. The plates were then incubated overnight at 35^{o}C. The antimicrobial inhibition zone sizes were then measured and interpretation done based on the CLSI guidelines.

3.10 PCR screening of β-lactamases genes

Isolates showing resistance towards third generation cephalosporin and meropenem were screened for carriage of *bla*$_{\text{NDM-1}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{TEM}}$ genes using the published primers, Table 3.1. Pure colonies sub-cultured on Mueller Hinton agar were used for DNA extraction using the boiling method. Briefly, the method involved heating bacterial inoculums suspended in 1mL of PCR water in an Eppendorf at 95 ^{o}C for 12 minutes. Separation was then done by centrifugation at 14000rpm for 5 minutes. The supernatant containing separated DNA was used as template for the PCR amplification. Reagents for PCR amplification were prepared as shown in Table 3.2. Fingerprint analysis using the $(GTG)_5$ primer was also done to determine the phylogeny of recovered *Pseudomonas aeruginosa* isolates. Amplified products were separated using 1.5 agarose gel and resultant bands visualized on gel max U.V imager.

Table 3.1: β-lactamases gene and finger printing primers.

Gene	Primer sequence (5'→3')	Annealing (T°C)	Reference
$(GTG)_5$	GTGGTGGTGGTG	40	(Mohapatra et al. 2007)
bla **$_{\text{SHV}}$**	**F**-TTATCTCCCTGTTAAGCCACC **R**-GATTTGCTGATTTCGCTCGG	50	(Hasman et al. 2005)
bla **$_{\text{TEM}}$**	**F**-GCGGAACCCCTATTTG **R**-ACCAATGCTTAATCAGTGAG	50	(Hasman, Mevius, Veldman, Olesen, & Aarestrup 2005)
bla **$_{\text{NDM-1}}$**	**F**-GAGATTGCCGAGCGACTTG **R**-CGAATGTCTGGCAGCACACTT	61	(Teo et al. 2015)

***bla*$_{SHV}$**: Sulhydryl β-lactamase variant, ***bla*$_{TEM}$:** Temoneria β-lactamase variant, ***bla*:** β-lactamase gene, ***bla*$_{NDM}$**: New Delhi β-lactamase, **bp**: molecular weight in base pair, **F**: forward primer, **R**: reverse primer.

Table 3.2: Table showing the preparation method of PCR for amplification

Reagents	**Calculated 23 µl final volume of one sample**
PCR master mix(containing buffer, taq polymerase, MgCL and dNTPs)	4 µl
PCR water	15 µl
Primer Forward	1 µl
Primer Reverse	1 µl
Betaine solution	1 µl
DNA	1 µl

The PCR results were then analyzed on 1.5 % agarose containing 0.3mg/l ethidium bromide and visualized under UVP Gelmax imager (Cicek *et al.,* 2014*)*

3.11 Ethical issues

Ethical approval from KEMRI IRB and Tigoni district hospital was obtained before the study commenced. Identified target population was introduced to the concepts and purpose of this study. The target population was informed that no monetary benefits were to be received for participation. A written consent was obtained from the study participants before sample collection and administering the questionnaire. For minor participants below the age of 18 years, their consent was first obtained before seeking written assent from the parent or guardian. The questionnaire was translated to the local Kikuyu language.

3.12 Data analysis

Statistical analysis of social-demographic characteristics in relation to *Pseudomonas aeruginosa* carriage and recorded antimicrobial resistance patterns was done using IBM SPSS version 20 software (SPSS, Inc. Chicago, IL). Chi-square test was used to calculate P-value for risk factors associated carriage of *Pseudomonas aeruginosa* in wound infections. Binary logistic regression analysis was carried out to generate the adjusted odds ratio with 95% confidence interval, an alpha of less than 0.05 ($P < 0.05$) was considered statistically significant.

3.13 Study limitations

The study was not able to check on other factors like education levels of the respondents. The possibility of use of counterfeit drugs was one area that the study could not delve into, this could be important in the misuse of carbapenems. The study did not also obtain information on whether the participants were diabetic, a health condition which has previously been associated with chronic wound infections. The study was not able to breakdown the type of the wounds and their anatomical sites.

CHAPTER FOUR

RESULTS

4.1 Analysis of *Pseudomonas aeruginosa* carriage in relation of sex and age

A total of 85 non-duplicate *Pseudomonas aeruginosa* strains were obtained from 299 wound cultures translating into a prevalence of 28%. Out of the 85 isolates recovered in this study, 80 (94%) were from adult participants while 5 (6%) were from minors (P: 0.001, C.I:2.1-14.0, O.R:3.4). Wounds in females were more colonised with *Pseudomonas aeruginosa* at 67% compared to those of males at 33% (P: 0.19, C.I:0.84-2.4, O.R: 1.42).

4.2 Factors associated with carriage of *pseudomonas aeruginosa* in wounds

Analysis of demographic characteristics revealed that majority of the participants obtains antimicrobials from a community chemist (60%) as opposed to hospital pharmacy (40%) Table 4.1. Although the study was not able to explain the relationship between source of drugs and wound colonization, patients who got their medication from community chemist were more likely to test positive for *Pseudomonas aeruginosa* compared to those who obtained drugs from hospital pharmacies (P: 0.001, C.1:3.-3.88, O.R:2.3)**.** Participants who purchased antimicrobials without a doctor's prescription regardless of the source of the drug were more likely to be colonized by a resistant strain compared to those to those who had a valid prescription (P: 0.001, C.I:3.01-8.86, O.R:5.17). Participants who did not complete dosage had a higher carriage (50.6%) of *Pseudomonas aeruginosa* compared to 49.4% those who that completed their dosage (P: 0.12, C.I:0.9-2.5, O.R: 1.5) though this was not significant

Table 4.1: Factors associated with carriage of *pseudomonas aeruginosa* in wounds

Category	Number with growth N (%)	Odds Ratio	95% Confidence Intervals	P-Value
Adults	80 (94.1)	3.4	(2.1-14.0)	0.001
Children	5 (5.9)			
Gender:				
Female	57(67.1)	1.42	(0.84-2.4)	0.19
Male	28(32.9)			
Source of antibiotic:				
Chemist	51 (60.0)	2.3	(1.39-3.88)	0.001
Hospital	34 (40.0)			
Prescription by doctor:				
No	52 (61.2)	5.17	(3.01-8.86)	<0.001
Yes	33 (38.8)			
Duration of antibiotic use:				
5 days	42 (49.4)		Chi Square= 9.23	
7 days	39 (45.9)			
10 days	4 (4.7)			
Completed dose:				
No	43 (50.6)	1.5	(0.9-2.5)	0.12
Yes	42 (49.4)			

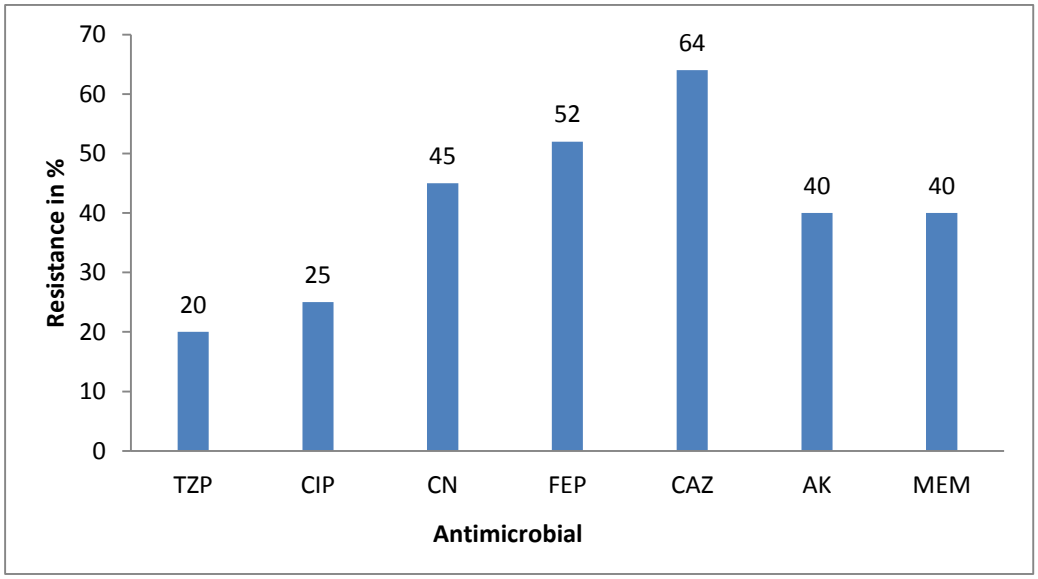
4.3 Antimicrobial susceptibility patterns of *Pseudomonas aeruginosa* isolates.

Among the 7 antimicrobial tested, highest resistance was recorded towards ceftazidime (64%), followed by cefepime (52%) while Piperacillin-tazobactam was least resisted (20%). Figure 4.1. *Pseudomonas aeruginosa* isolates were more resistant towards gentamicin (45%) and amikacin (40%) compared to ciprofloxacin (25%). High resistance towards imipenem was also recorded in a high number of isolates (40%).

Table 4.2: Antimicrobial Inhibitions Zones of Pseudomonas aeruginosa isolates.

Antimicrobial agent	Zone diameter in Millimeter (MM)Interpretative criteria			
	S	No of Isolates(n-85)	R	No of Isolates(n-85)
Ciprofloxacin	≥ 16	63	≤ 15	22
Gentamicin	≥ 13	46	≤ 12	39
Amikacin	≥15	51	≤14	34
Meropenem	≥16	51	≤15	34
Tazobactum-piperacillin	≥15	68	≤14	17
Ceftazidime	≥15	31	≤14	54
Cefepime	≥15	40	≤14	45

Plate 4.1: Image of a Muller Hinton plate showing Inhibition zones



Key: TZP: Tazobactum-piperacillin, **CIP**: Ciprofloxacin, **CN**: Gentamicin, **FEP**: Cefepime, **CAZ**: Ceftazidime, **AK:** Amikacin, **MEM:** Meropenem.

Figure 4.1: Antimicrobial resistance patterns of *Pseudomonas aeruginosa* isolates from wound samples

4.4 Antimicrobial resistance patterns in relation to Sex and Age

Pseudomonas aeruginosa from females participants were resistant to aminoglycosides (AK 80%, CN 41%) compared to those from males counterparts (AK 79%, CN 38%). However resistance towards cephalosporins was higher in isolates from male participants to females where resistance towards CAZ was 68% against 53% and FEP was 53% and against 51%. Isolates from participants aged between 18-30years were overall more resistant compared with those obtained from other age brackets Table 4.3 A higher resistance towards virtually all tested antimicrobial agents was also recorded in bacterial isolates from participants who did not complete dosage compared to those that did.

Table 4:3: Antimicrobial resistance patterns of *Pseudomonas aeruginosa* isolates in relation to Sex and Age of the study participants.

		Resistance %						
	n	**CN**	**TZP**	**CIP**	**FEP**	**CAZ**	**AK**	**MEM**
All	85	45	20	25	52	64	40	40
Gender of source								
Males	34	38	21	26	53	68	79	59
Females	51	41	20	24	51	67	80	73
Age of source								
Children <13 years	5	20	0	0	60	80	100	80
Children 13-17	3	33	0	0	0	0	100	0
Adults 18-30	35	51	34	37	66	83	83	83
Adults 31-50	25	24	8	12	36	52	68	52
Adults >50	17	47	18	29	53	71	82	65
Prescription adherance								
Dose completed	4	0	0	0	75	100	75	75
Dose incompleted	81	42	21	26	51	67	80	67
No prescription	66	44	23	24	47	65	79	64
with prescription	19	26	11	26	68	79	84	79
Source of drugs								
Drugs from hospitals	24	29	17	29	75	79	83	79
Drugs from chemist	61	44	21	23	43	64	79	62

TZP: Tazobactum-piperacillin, CIP: Ciprofloxacin, CN: Gentamicin, FEP: Cefepime, CAZ: Ceftazidime, AK: Amikacin, MEM: Meropenem, n: total count of corresponding feature.

4.5 Carriage of ß-lactamases genes

Carriage of bla_{TEM} (24.7%) was common in most in *Pseudomonas aeruginosa* with most of these isolates showing resistance towards ceftazidime. Carriage of bla_{SHV} (22%) was also common among isolates resistant ceftazidime, gentamicin and cefepime. bla_{NDM} was detected in 10 (12%) isolates that were resistant to ceftazidime, cefepime, amikacin and meropenem, Figure 4.2.

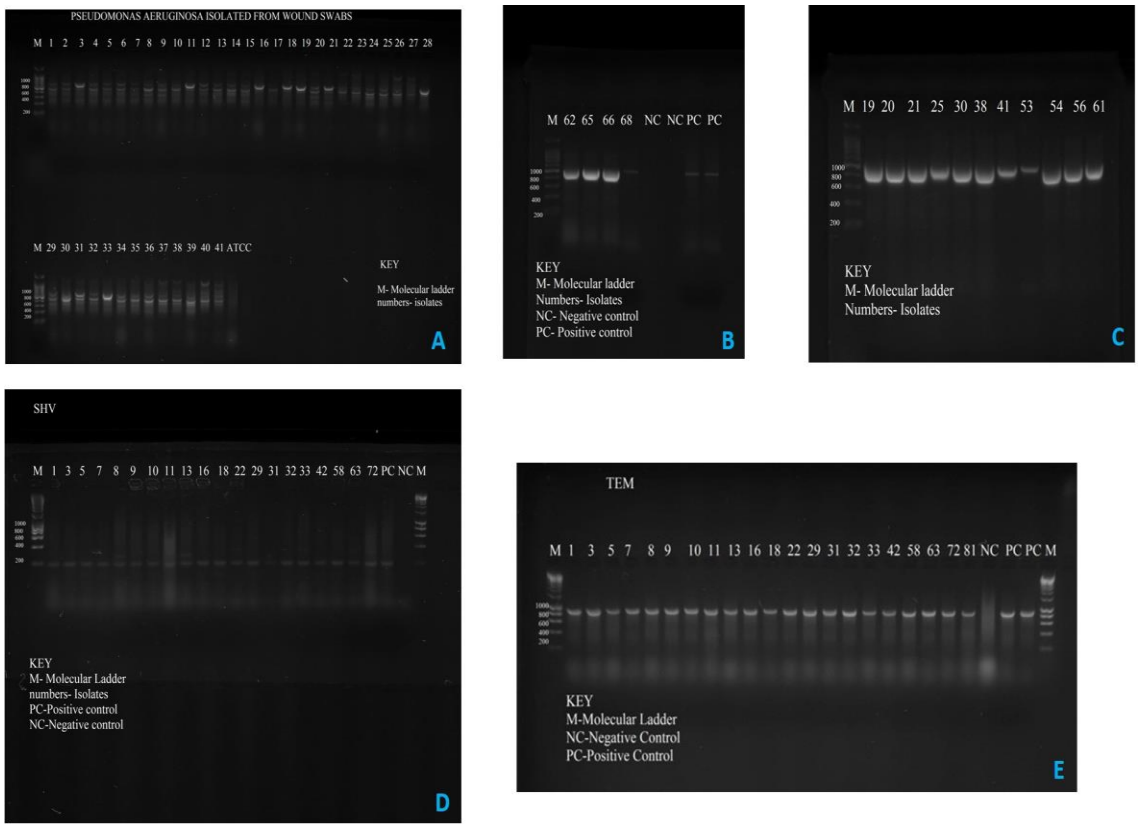


Figure 4. 2: Gel images for screened resistance genes

Key

A- (GTG)5 fingerprint analysis image. The image shows banding image of 41 analyzed *Pseudomonas aeruginosa* isolates.

B- Gel image for bla_{NDM}. The image shows 4 positive isolates, negative and positive control strain.

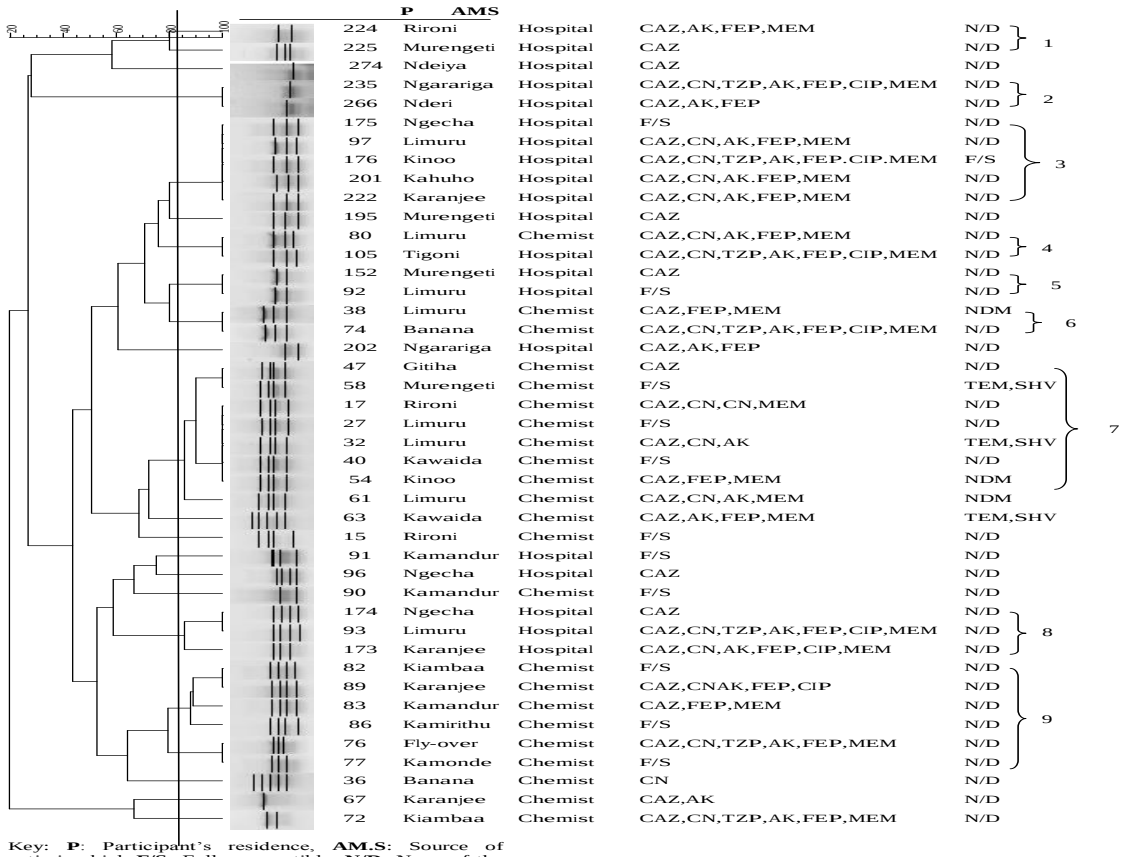
C- Gel image for bla_{NDM} gene

D- Gel image for bla_{SHV} positive in 20 isolates, a negative control (N.C), positive control (P.C) and 100kb molecular ladder.

E- Gel image for bla_{TEM} positive in 21 isolates, a negative control (N.C), positive control (P.C) and 100kb molecular ladder.

4.6 Genetic relatedness of recovered *Pseudomonas aeruginosa* isolates

Tight clustering was noted in *Pseudomonas aeruginosa* isolates from diverse patients visiting Tigoni District Hospital. A similarity of ≥ 80% was also noted in 9 clusters based on isolates banding patterns, participant area of residence, and source of antimicrobial agents, resistance phenotype and genotype. Besides the 9 highly clonal strains, most isolates had varying resistance phenotypes and genotype which perhaps is an indication that different clones have acquired these resistances independently, Figure 4.3.



Key: **P**: Participant's residence, **AM.S**: Source of antimicrobial, **F/S:** Fully susceptible, **N/D**: None of the target gene was detected

Figure 4.3: Phylogeny of recovered *Pseudomonas aeruginosa* isolates

Figure 4.3: Phylogeny od recovered *Pseudomonas aeruginosa* isolates

CHAPTER FIVE

DISCUSSION

Pseudomonas aeruginosa is one of the leading cause of soft tissue infections, including surgical and chronic non-traumatic wound infections especially in immunocompromised individuals (Siddiqui & Bernstein, 2010). Although surgical wounds are the most prevalent, non-traumatic wounds are a significant cause of mortality. The current study, recorded a prevalence of 28% in wound carriage of *Pseudomonas aeruginosa* which lower than previously reported in tertiary Nigeria (85.05%) perhaps due to difference in study settings. The age of the study participants seems to have a bearing in wound infections where elderly were significantly colonized which in constituent with findings of previous studies conducted in Nigeria and Ethiopia (Azene & Beyene, 2011; Egbe, *et al*., 2011; Siddiqui & Bernstein, 2010). Although the study did not establish the association between bacteria colonization and environmental risk factors, *Pseudomonas aeruginosa* is ubiquitous in environments and therefore open wounds are exposed to contamination that may emanate from soil among other sources. Although the study also did not establish whether recruited patients were diabetic, most cases on chronic non-traumatic wound infections have previous been associated with Diabetes mellitus due to the immune-compromised status (Neves *et al.,* 2019). *Pseudomonas aeruginosa* accounts for approximately 11% of these cases in developed countries often associated with limbs amputation and high mortality (Baumfeld, *et al.,* 2018), but this data is limited in developing countries such as Kenya.

Most of the isolates showed high resistance towards tested antimicrobial agents showed resistance above 20%. A previous study conducted in Kenya by Mukaya *et al.,* (2018), reported higher resistances of more than 40% to most antimicrobial agents in blood, respiratory tract, urine and burn wound *P.s aeruginosa* isolates. Among them is the resistance rate of 50.5%, 52.7% and 67.6% towards TZP, CIP and MEM respectively which is higher than the current study. Although aminoglycosides, β-lactams and fluoroquinolones are the most effective antipseudomonal agents, the high resistance

recorded to these classes of antimicrobial in most bacteria strains that were recovered which suggest that the resultant wounds infections are difficult to treat. Resistance to carbapenem (MEM 40%) further diminish the available treatment options increasing chances of prolonged healing process, hospitalization and mortality in chronic cases especially in immunocompromised patients. The study however showed that tazobactam and ciprofloxacin are effective antipseudomonal agents in wound treatments which is contrary to findings of previous study in Kenya by Mukaya *et al.,* (2018).

The study found out that use of un-prescribed and non-completion of antimicrobial dosage stands out as significant risks for development of antimicrobial resistance. This is linked to the finding that most patients sourced their antibiotics from community chemists which suggests self-medication. Self-medication is characterized by use of antibiotics without a prescription and unsupervised use often leading to non-completion of dosage. The high poverty levels may cause some patients not to afford the complete dose. Findings from other studies show that the unrestricted and indiscriminate use of antimicrobials, over-the-counter sale, self-medication, consumption of counterfeit drugs or low cost generics, improper dosage and non-adherence to dosage are very common and fuel the process of positive selection for many types of antibiotic resistance leading to high multidrug resistance. (Tolbert *et al.,* 2016, Satyajeet *et al.,* 2014, Manenzhe *et al.,* 2015 Chika *et al.,* 2017, Chander *et al.,* 2013 , Magiorakos *et al* ., 2011*)* .

The study reported a prevalence of 21%, 20% and 10% in *Pseudomonas aeruginosa* carriage of $bla_{\text{TEM,}}$ bla_{SHV} and bla_{NDM} respectively. This is the first report of $bla_{\text{NDM-1}}$ in *Pseudomonas aeruginosa* isolates from rural hospital in Kenya. This therefore is an indication of spread of this gene to a rural area which is proximal to the capital centre of Nairobi where the first case of $bla_{\text{NDM-1}}$ in *Klebsiella pneumoniae* was reported in 2011 (Poirel *et al.,* 2011). $bla_{\text{NDM-1}}$ and $bla_{\text{NDM-1}}$ have been previously reported in Kenyatta National Hospital by Mukaya *et al* (2018) in wound, blood , urine and respiratory aspirates *Pseudomonas aeruginosa* isolates. Increased travel and migration plays a role in this spread. The $bla_{\text{NDM-1}}$ confers resistant to multiple antimicrobials and the study found that in all the cases the gene was present the isolates were resistant to multiple

drugs. Most remarkably all these isolates were resistant to meropenem. The transfer and spread of $bla_{\text{NDM-1}}$ $bla_{\text{TEM,}}$ bla_{SHV} among other resistance genes if unchecked will limit the repertoire of drugs available for treatment of multidrug resistant *Pseudomonas aeruginosa* wound infections. This has a negative impact on the management of wound infections especially among rural people most of whom cannot afford high cost of proper and advanced treatment options which further exacerbates the situation.

The phylogeny showed 9 tight clusters among most NDM *Pseudomonas aeruginosa* isolates. Although $(GTG)_5$ is not a very powerful phylogeny method, there is possibility that the NDM producing strains are clonal going by their clustering and resistance profiles. Therefore, there is a possibility of clone stability of the highly resistant NDM strains. The study also noted that the non-NDM strains did not cluster tightly, possibly indicating independent evolution under the influence of distinct resistance pressures. Although the study was not able to determine possible carriage of genetic elements such as plasmids that could possibly cause above mention resistance features, further genetic analysis and SNP typing based on WGS data will shed light into this. Clonal spread of NDM producing strains that are also associated with high resistance non-carbapenem antimicrobial is therefore a serious health risk in development of difficult to treat infections. The spread of clonal strains in clinical set-up has been associated with use medical devices such as catheters and prosthetics (Aiken *et al.,* 2011). *Pseudomonas aeruginosa* strains are able to survive in the environment especially in most areas. Therefore, person to person transmission is also possible especially in contaminated environment. There should therefore be deliberate effort to reduce transmission and source reservoir of such resistance strains in such settings.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusions

i. Open wounds of many patients observed in this study make such infections from colonization of bacterial strains that may emanate from the environment. *Pseudomonas aeruginosa* is ubiquitous in the environment compartments such as contaminated surfaces and soil. Most residents are farmers and casual workers; therefore, the risk of injuries associated with these occupations further increases the risk of wound colonization by bacterial strains.

ii. The antimicrobial susceptibility of 80-75% by Tazobactam/Pipperacilin and Ciprofloxacin shows they are ideal anti-pseudomonal agents.

iii. Self-medication is a is a risk factor as it involves use of antibiotics without prescription and non-completion of dosage which is associated with bacterial carriage and acquisition of resistance

iv. There is emergence of $bla_{\text{NDM-1}}$ in a rural area linked to widespread misuse of Carbapenems which is alarming.

v. The clonal stability of *Pseudomonas aeruginosa* strains producing $bla_{\text{NDM-1}}$ and diversity of non- $bla_{\text{NDM-1}}$ producing strains indicates independent acquisition of resistance.

6.2 Recommendations

i. Measures to institutionalize antimicrobial stewardship such as hospital committees, prescription guided by empirical laboratory susceptibility patterns and continued monitoring of development of resistance should be initiated. It will be important to leverage on the National Anti-Microbial Resistance plan, by strengthening the infection control committee and sharing information with clinicians. It is hoped that this information will assist to minimise the risk of

development of resistance by ensuring the use of correct drugs, dosing and combination therapy

ii. Measures to closely monitor dosage completion and post –treatment follow-up for serious infections should be initiated where possible to prevent emergence of antimicrobial resistance.

iii. It is common for people to purchase antimicrobials without prescription. Most people use antimicrobials and do not complete the recommended doses. Relevant bodies should ensure rational use of antimicrobials by restricting over the counter sales of antimicrobial agents with prescription. There should be enhanced antimicrobial stewardship, by adopting measures that will improve antibiotic prescribing practices and stop the excessive indiscriminate use of antimicrobials.

iv. Only a few surveillance studies have been conducted in central region of Kenya. Perhaps more surveillance studies in future should also give focus to rural settings in effort to improve human health through infections risks assessment and better treatments optionsIn Kenya, most studies conduct have reported few genetic mechanisms that can be attributed to resistance in β-lactams and carbapenems. Future studies should therefore use high resolution methods such as whole genome sequencing (WGS) to shed more light on full mechanisms of resistance in *Pseudomonas aeruginosa* isolates in the region.

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APPENDICES

Appendix I: Role of Investigators.

Thomas Gachuki Thuo is a Master of Science Medical Microbiology student at JKUAT/KEMRI here and referred to as the Principal Investigator for this work. He is registered with the Kenya Medical Laboratory Technologist and Technicians Board. Registration number: A0088. He conducted research based on the procedures in this protocol as part of his Degree requirement.

Dr. John Kiiru is a researcher at KEMRI, Centre for Microbiology Research and is the co-investigator in this project. He is also a part time lecturer at JKUAT. He gave technical advice to the PI.

Prof. Ciira Kiiyukia is a Lecturer at the Institute of Tropical Medicine and infectious Diseases (JKUAT) and a co-investigator. He provided the research team with a key link between JKUAT and KEMRI administration. He also provided technical assistance and advice.

Appendix II: List of materials

1. Sheep blood agar
2. MacConkey agar 500g
3. Oxidase reagent
4. Glucose fermentation
5. Lysine hydrolysis
6. Screw cap, universal urine containers
7. Screw cap, universal sputum containers
8. Sterile swabs
9. Petri-dishes
10. Non-sterile gloves
11. Mueller-Hinton 500g Agar
12. LPG gas cylinder
13. Yeast extract 250g
14. PCR machine
15. Primers -

Appendix III: Culture media preparation

Mueller-Hinton Agar

1. Prepare MHA from a commercially available dehydrated base according to the manufacturer's instructions.
2. Immediately after autoclaving, allow the agar to cool in a 45 to 50°C water bath.
3. Pour the freshly prepared and cooled medium into glass or plastic, flat-bottomed Petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 60 to 70 mL of medium for plates with a diameter of 150 mm and 25 to 30 mL for plates with a diameter of 100 mm.
4. Allow the agar plates to cool further to room temperature and, unless the plates are used the same day, store in a refrigerator (2 to 8°C).
5. Use the plates within seven days after preparation unless adequate precautions, such as wrapping in plastic, are taken to minimize drying of the agar.
6. A representative sample of each batch of plates should be examined for sterility by incubating at 35 ± 2°C for 24 hours or longer.
7. Check the pH of each batch of MHA when the medium is prepared. The agar medium should have a pH between 7.2 and 7.4 at room temperature, and must therefore be checked after gelling.
8. Do not add supplemental calcium or magnesium cations to MHA.

0.5 McFarland Turbidity Standard

1. Prepare a 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2 \cdot 2H_2O$) stock solution.
2. Prepare a 0.18 mol/L (0.36 N) H_2SO_4 (1% v/v) stock solution.
3. With constant stirring to maintain a suspension, add 0.5 mL of the $BaCl_2$ solution to 99.5 mL of the H_2SO_4 stock solution.
4. Verify the correct density of the turbidity standard by measuring absorbance using a spectrophotometer with a 1-cm light path and matched cuvettes. The absorbance at 625 nm should be 0.08 to 0.13 for the 0.5 McFarland standard.
5. Transfer the barium sulfate suspension in 4- to 6-mL aliquots into screw-cap tubes of the same size as those used for standardizing the bacterial inoculum.

6. Tightly seal the tubes and store in the dark at room temperature.
7. Vigorously agitate the barium sulfate turbidity standard on a vortex mixer before each use and inspect for a uniformly turbid appearance. Replace the standard if large particles appear. Mix latex particle suspensions by inverting gently, not on a vortex mixer.
8. The barium sulfate standards should be replaced or their densities verified monthly.

MacConkey Agar without salt

1. 47 g of the medium was suspended in one litre of purified water.
2. Heating with frequent agitation was done and boiling for one minute to completely dissolve the medium.
3. This solution was autoclaved at $121^{O}C$ for 15 minutes.
4. Media was poured into petri-dishes and allowed to cool.
5. Media will then be packed and stored refrigerated at $4^{O}C$ for future use.

Blood agar preparation

1. Measure 500ml of distilled water using a measuring cylinder and transfer into a 1 litre conical flask.
2. Weigh 20g of Blood Agar Base (SBA) using a weighing balance.
3. Suspend the measured SBA into the 500ml of distilled water.
4. Mix thoroughly (dissolving occurs during autoclaving).
5. Autoclave at 121°C for 15 minutes.
6. Allow the autoclaved SBA to cool to 45-50°C and then aseptically add 25ml of sterile defibrinated blood. Mix thoroughly.
7. Arrange the petri-dishes onto the clean safety hood and then gently pour the warm blood agar onto the plates.
8. Using a bunsen burner gently invert and pass the flame over the poured blood agar in the plate such that the air bubbles are removed.

9. Cover the petri-dishes and allow the blood agar to coagulate before storage in a refrigerator.

Appendix IV: Questionnaire

Title of Research: Cross Section Study On Susceptibility Patterns And Presence Of resistance Genes In *Pseudomonas Aeruginosa* Infections In Tigoni District Hospital In Kenya Investigators: Thomas Gachuki Thuo: Jomo Kenyatta University of Agriculture and Technology, John Kiiru: KEMRI-CMR, Ciira Kiyuukia: JKUAT

Date : ...../ ...../ 20……. Barcode ID : .....

Patient Hospital No…… Age……

Gender: (Tick where appropriate) Male ☐ Female ☐

Place of residence: .....

HISTORY OF ANTIBIOTIC USE

1. Last time antibiotic was used……
2. Where did you get the antibiotic from……Hospital ☐ivate chemist ☐
3. Was it a doctor's prescription……..Yes ☐ No ☐
4. How long was antibiotic used…….
5. Did you complete full dose of the antibiotic Yes No
6. If no for no(5) above state why?

Type of Specimen

WOUND SWAB ☐

Appendix V: Consent forms

PATIENT WRITTEN CONSENT FORM

The study you are about to participate in is on drug susceptibility patterns and presence of resistance genes in the infections caused by a bacteria called *Pseudomonas Aeruginosa.* The duration of the study will be 12 months and no follow up. You will only give one sample/specimen. Should you agree to participate in the study, you will be asked to allow a trained person to collect a wound swab from a wound on your body.

All data collected from you will be coded in order to protect your identity. We will not use your name but only use your hospital number. Only the research study staff will have access to the information. At the end of the study, there will be no way to link your name with your data. Any additional information about the study will be provided to you including the final study results.

You are free to withdraw or refuse to answer any questions at any time without any consequences.

Should you agree to participate in the study, please sign your name below, indicating that you have read and understood the nature of the study, your responsibilities as a study participant, the inconveniences associated with voluntary participation in the study and that all your questions and concerns concerning the study have been answered satisfactorily. You understand Jomo Kenyatta University and KEMRI has no policy or plan to pay for any injuries you might receive as a result of participating in this research protocol

You will receive a copy of this signed consent form to take away with you.

_____

_____

Signature of Study Participant and Date Thumbprint of Study Participant and Date _____

Signature of Person Obtaining Consent and Date _____

Signature of Witness and Date

The participant received a copy ☐

PATIENT WRITTEN CONSENT FORM IN KIKUYU

Uthuthuria uyu ukonie germs muthemba wa bakteria itagwo pseudomonas aeruginosa. Tura thima dawa mithemba miingi nakurora njira iria bacteria ino ihotaga gwithema dawa icii. Ututhuria uyu ni wa mieri ikumi na iri tu. Wee wita umwe uria ugukoro uthuthuriaini uyu ukuheana githimo gia kironda.

Tutikuhuthira ritwa riaku, tukuhuthira naba yaku ya thibitari tu. Tukuhe githimi giaku namba ati no aria mareka uthuthuria uyu mangimimenya. Gutiri mundu ungihota kumenya riatwa riaku kana kumenya niwee waheanire githimi kiu.

Wina rutha kana wiathi wa kwieheria kuma gwi uthuthuria uyu kana kwaga gucookia kiuria ogiothe .

Weetikira gukorwo wi thiini wauthuthuria uyu, ugwikira thairi kana kirore fomu ino. Uguo nikuga ati niwienderi waku mwene.

Ni ukuheo fomu ota ino wiigire.

_____ _____

Thairi yaku na tareki kana kirore giaku na tareki

_____

Thairi ya muthuthuria na tareki

_____

Thairi ya muira na tareki

Mhusika niaheo copy ya fomu? ☐

CONSENT TO STORE BACTERIA ISOLATES

Title: Cross Section Study On Susceptibility Patterns And Presence Of *Aac (6')-Ib-Cr* Genes In *Pseudomonas Aeruginosa* Infections In A Rural Hospital In Kenya

Storage: *Pseudomonas aeruginosa* isolates from this study will be stored in Tryptic soy broth with 15% glycerol at -80^{O}C for future studies relevant infections and antimicrobial resistance for a period of not more than 5 years.

Rationale for future tests

- For comparison with other phenotypic multi-drug resistant *Pseudomonas aeruginosa* isolates from other regions of the world.
- For molecular sequencing of drug resistance genes present the isolates.

BARCODE 1.D

LAB I.D: ……..

Participant Consent

1. "Will you allow me to take your wound swab sample for testing in the laboratory?"

Yes …….. 1

No …… 2

2. "Will you allow us to keep bacteria isolates got from your sample for later testing?"

Yes ……..... 1

No ……... 2

3. "Will you allow us to ship specimens to Welcome trust laboratory in the United kingdom for any testing?"

Yes ……..... 1

No ……... 2

Date…./……/…… Participant Signature: ……Signature of Witness: ……..

Lab Tech Name/ ID: …….

Signature of Laboratory Technician: …….....

CONSENT TO STORE BACTERIA ISOLATES in Kikuyu

Rutha rwa kuiga ithimi gwa kahinda.

Kiongo gia uthuthuria: Mirimu iria irehagwo ni bacteria muthembawa *Pseudomonas Aeruginosa* thiini wa aruaru thibitariini ya Tigoni.

Kuiga bacteria barabuini nauhehuwa-$80^{O.}$ Ithimi ici niikahuthika nja ya bururi kuria ruraya gwa kahinda gatakiritie miaka itano.

Gitumi gia kuiga

- Kuhanania maumithio na mabururi mangi.
- Guthima kihuumo kana mbari cia bacteria iyo.

namba ya thiri

namba ya lab: ……..

Ruutha rwa gukorwo uthuthuria ini uyu.

4. "Niwetikira ndware githimigia kironda, laboratory?"

Nindetikira…….. 1

Nindarega…… 2

5. Niwetikira njige bakteria icio gwa ihinda ria miaka itano niguo ithiimi ingi igathimwo?

Nindetikira…….. 1

Nindarega…… 2

6. "Niwetikira ndume bacteria bururini wa ruraya(Welcome trust laboratory United kingdom) ?"

Nindetikira…….. 1

Nindarega…… 2

Tareki…./……/……

Thairi ya mhusika: …….

Thairi ya muira: ……..

Lab Tech Name/ ID: …….

Signature of Laboratory Technician: …….....

Appendix VI: Published Manuscript

Scientific Research Publishing

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Antimicrobial Resistance Profiles and Clonal Relatedness of *Pseudomonas aeruginosa* Strains Recovered from Wounds Infections of Outpatient Population Presenting in a Rural Hospital in Kenya

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Abstract

Pseudomonas aeruginosa is a leading cause of hospital infections and is intrinsically resistant to most antibiotics. Emergence of multidrug resistant (MDR) strains has been reported in the world and poses a great challenge in the management of infections associated with this species. While a substantial amount of research has been done on strains from most of other infection caused by this species in developed countries, little is known about the susceptibility profiles of strains recovered from African countries in general and Kenya in particular. Furthermore, there is paucity of data regarding strain, phenotype and genetic diversity of strains recoverable from wounds among patients in Kenya. The possible risk factors for acquisition of MDR strains and possible factors that could fuel clonal expansion in hospital and community settings remain undetermined. This cross-sectional study conducted in Tigoni Hospital, a rural area in Central Kenya sought to determine risk factors associated with carriage of MDR *Pseudomonas aeruginosa* in wounds among rural population. We also analyzed antimicrobial resistance profiles among these isolates. Prevalence of *P. aeruginosa* in wounds was 28% with 85 isolates recovered from wounds of 299 participants. Most antimicrobial resistance prevalence was recorded towards Ceftazidime (64%) and Cefepime (52%) while Piperacillin-tazobactam was the most effective antimicrobial agent with a resistance prevalence rate of 20%. Resistance towards new classes of aminoglycosides such as Gentamicin was at 45% while that towards Amikacin was at 40%. Compared to other related studies, relatively lower resistance towards Ciprofloxacin (25%) and Meropenem (40%) were recorded. Some

of the risk factors identified for carriages of MDR strains were self-medication (p: 0.001, C.I: 3.01 - 8.86, O.R: 5.17) and non-completion of dosage (p: 0.12, C.I: 0.9 - 2.5, O.R: 1.5).

Keywords

Carbapenems Resistance, Risk Factors Related to Carbepenems Resistance, Carriage of *Pseudomonas aeruginosa* in Wounds, Clonal Relatedness

1. Introduction

We detected bla_{NDM-1} in 10 isolates from a total of 34 isolates that were resistant to Meropenem. We were not able to determine genetic basis of resistance to this class of antimicrobials in the rest 24 isolates but these isolates have since been submitted for whole genome sequencing. bla_{TEM} was the most prevalent β-lactamase gene at 25% while the prevalence of bla_{SHV} was (24%) was also recorded amongst *strains resistant to 3rd generation cephalosporins.*

The phylogeny of recovered *strains* revealed significant genetic similarities among the strains. Our data revealed possible clonal expansion of some MDR strains. However tight clustering of strains that bore dissimilar resistance phenotypes further suggests independent acquisition of a similar set of resistance determinants among strains belonging to different clones. These isolates tested negative for other carbapenemases such as bla_{VIM}, or even KPC. This is the first report of carbapenemases resistant *Pseudomonas* from skin wounds in Kenya. The close relatedness phenotypically further indicates clonal expansion of these carbapenem resistant strains in the community. Unless something is done to curtail the dispersal of these strains, they could soon be implicated in large outbreaks in community and hospital settings thereby rising mortality and morbidity due to their insensitivity to majority of available drugs.

2. Background

Among the *Pseudomonas* species, *Pseudomonas aeruginosa* is the most important clinically. It is a Gram-negative rod ubiquitous in hospital environment especially in hot tubs, whirlpools, contact lens irrigation fluids, aerators, sinktraps, showers, and respiratory equipment. *P. aeruginosa* has been implicated in severe infections in immune-compromised individuals ranging from bacteremia, urinary tract infections, wound, respiratory, skin and burn infections. [1] [2] In the repertoire of infections caused by the organism wound infections come out as most common and therefore, the organism is a leading cause of life threatening wound infections often with high mortality rates [3]. This is in both chronic and acute wounds where several virulence factors come into play including the formation of biofilms. Factors such as loss of protection offered by intact epidermis through burns, puncture, wounds and eye trauma provide ideal condition for *P. aeruginosa* infections especially in hospital wards. Intrinsic resistance to multiple

antibiotics in addition to formation of biofilms makes control and treatment of this organism difficult [4] [5]. This organism is able to thrive well in moist environment and therefore clonal spread of could cause of persistent nosocomial infections in the hospital environment. Indeed, previous studies in Kenya have documented *P. aeruginosa* to be the predominant cause of nosocomial infections in local hospitals [6]. In a study conducted in Agha Khan Hospital between 2006 and 2007, 57 Carbapenem resistant *Pseudomonas aeruginosa* isolates were obtained from blood, urine, respiratory tract and wound specimens. Notably 30% of these carbapenem resistant *P aeruginosa* were obtained from wound specimens.

Antimicrobial resistance in *P. aeruginosa* has largely been attributed to production and acquisition of extended β-lactamases. These resistance mechanisms are mostly harbored in plasmid borne integron and are therefore easily transmissible across species and genera *via* horizontal gene transfer [7] [8]. Genetic elements such as Class 1 and 2 integron conferring resistance to β-lactams, aminoglycoside and fluoroquinolones have also been reported in *P. aeruginosa*. These classes of antimicrobials have high applicability in treatment of *P. aeruginosa* infections. Production and spread of carbapenemases that degrade carbapenems which are regarded as some of the drug of last resort for treating Gram-negative bacterial infections is on the rise among *P. aeruginosa*. This therefore makes treatment of associated infections more difficult and likely to result to treatment failure that could in turn result to prolonged hospitalization, high morbidity, and mortality.

Prevalence and antimicrobial resistance profiles of *P. aeruginosa* in rural Kenya have not been adequately studied. In urban centers, considerable self-medication and unhygienic environment have been among the major causes of antimicrobial resistance in such settings. In rural set-up on the other side, the drivers to this antimicrobial resistance have not been well documented. This cross-sectional study therefore set to determine prevalence of wound infections caused by *P. aeruginosa* and antimicrobial resistance in Tigoni hospital which is among tertiary hospital in Kiambu county attracting referrals within and beyond.

3. Methodology

1) Study area and design

A consecutive random sampling strategy was used in this cross-sectional study to obtain wound and swab specimen from consenting patients attending Tigoni District hospital for a period of six months in 2015. In total, 299 participants were recruited in this study where 299 of wound/swabs specimens were obtained. Of these specimens 240 (80%) were from adults and 59 (20%) were from children. Further, 183 (61%) were obtained from males compared to 116 (39%) from females. The mean age was 33.6 years and median age 29 years.

2) Sample collection

Patients with wounds were identified at the outpatient department, medical

and surgical wards. A wound and/or pus swab obtained from the ailing participants was transported in Cary-Blair media in a cool box to the National Microbiology Reference Laboratory within three hours of collection. Unique codes were assigned to sample collection forms were transported along with the collected barcode samples. Social demographic data was captured included patient's age, gender, residence, antimicrobial source and ease in accessing over the counter, prescription availability and dose completion was captured on a structured questionnaire.

3) Bacteria isolation and identification

Pus and wound swabs were directly plated on sheep blood agar (SBA), Mueller Hinton agar and MacConkey with salt and incubated at 35˚C, for up to 24 hours. On MacConkey agar plate, non-lactose fermenters colonies with characteristic dry translucent edges were presumed as *P. aeruginosa*. On SBA, *P. aeruginosa* colonies were shiny, dry with rough edges, β-hemolytic with grape like smell. Identification was done using Gram stain and a series of biochemical tests such as Oxidase (positive), glucose fermentation, hydrolysis of arginine, and nitrate production test as previously described [9].

4) Antimicrobial Susceptibility Testing

Antimicrobial susceptibility tests were performed using Kirby–Bauer disc diffusion technique on Mueller Hinton agar (Oxoid). A McFarland turbidity standard of 0.5 was evenly spread on Mueller Hinton plate to obtain a confluent lawn. Antimicrobial discs included; Gentamicin (GEN 10 µg), Amikacin (AMK 30 µg), Ciprofloxacin (CIP 5 µg), Piperacillin-tazobactam (TZP 100/10 µg), Ceftazidime (CAZ 30 µg), Cefepime (CPM 30 µg) and Meropenem (MEM 10 µg) were then dispensed. *P. aeruginosa* ATCC strains ATCC 27853 was used as control strain for media quality and disc potency. The plates were incubated overnight at 35˚C. The antimicrobial inhibition zone sizes were then measured and interpretation done based on the CLSI guidelines 2016.

5) PCR screening of β-lactamase genes

Isolates showing resistance to third generation cephalosporin were screening possible carriage of $bla_{\text{NDM-1}}$, bla_{SHV} and bla_{TEM} genes which have are the most reported variants in the region. Pure colonies sub-culture on Mueller Hinton agar were used for DNA extraction using the boiling method. Briefly, the method involved heating bacterial inoculums suspended in 1000 µl of PCR water in an Eppendorf at 95˚C for 12 minutes. Separation was then done by centrifugation at 14,000 rpm for 5 minutes. The supernatant containing separated DNA was template for the PCR process. **Table 1** Finger print analysis using the $(GTG)_5$ primer done to determine genetic relatedness of recovered *Pseudomonas aeruginosa* isolates. Amplified products were separated using 1.5 agarose gel and resultant bands visualized on gel max U.V imager.

4. Results

1) Analysis of risk factors in relation to *Pseudomonas aeruginosa* carriage amongst study participants

Table 1. β-lactamase gene and finger printing primers.

Gene	Primer sequence (5' : 3')	Annealing (T°C)	Reference
$(GTG)_5$	GTGGTGGTGGTG	40	[10]
bla_{SHV}	F-TTATCTCCCTGTTAAGCCACC R-GATTTGCTGATTTCGCTCGG	50	[11]
bla_{TEM}	F-GCGGAACCCCTATTTG R-ACCAATGCTTAATCAGTGAG	50	[11]
bla_{NDM-1}	F-GAGATTGCCGAGCGACTTG R-CGAATGTCTGGCAGCACACTT	61	[12]

bla_{SHV}: Sulhydryl β-lactamase variant, bla_{TEM}: Temoneria β-lactamase variant, *bla*: β-lactamase gene, bla_{NDM}: New Delhi β-lactamase, bp: molecular weight in base pair, F: forward primer, R: reverse primer.

A total of 85 non-duplicate *P. aeruginosa* strains were obtained from successful MacConkey and sheep blood cultures. Carriage of *P. aeruginosa* was high in wounds of adult (94%) compared to those of children (6%) with a significant statistical difference (P: 0.001, C.I: 2.1 - 14.0, O.R: 3.4). Similarly, wounds in females were more likely to be colonized at 67% compared to those of males at 33% (P: 0.19, C.I: 0.84 - 2.4, O.R: 1.42).

Analysis of administered questionnaire further revealed that majority of the participants obtains antimicrobials from a community chemist as opposed to hospital pharmacy attributed to cost effectiveness. Carriage of *P. aeruginosa* was predominant among participants who obtained antimicrobial from community chemist (60%) compared to those who acquired the same from hospital pharmacies (40%) indicating that the source of antimicrobials determines the chances of colonization with this pathogen (P: 0.001, C.I: 3.01 - 8.86, O.R: 5.17).

Those who acquired antimicrobial without a doctor's prescription regardless of the source drug source were more likely to be colonized by a highly resistant strain compared to those to those had a prescription, P: 0.001, C.I: 3.01 - 8.86, O.R: 5.17. Participant who recorded did not finish their antimicrobial dose had a higher carriage of these bacteria (50.6%) compared to those who completed their dose (49.4%) further indicating that adhering to dosage regimen is an important risk factor for colonization with this pathogen (P: 0.12, C.I: 0.9 - 2.5, O.R: 1.5).

2) Antimicrobial susceptibility patterns of *Pseudomonas aeruginosa* isolates

Among the antimicrobial tested, highest antimicrobial resistance was recorded towards Ceftazidime (64%) whilst Piperacillin-tazobactam (80%) was the most effective in-vitro (**Figure 1**). Gentamicin (45%) was the least potent antimicrobial amongst the aminoglycoside tested. *Pseudomonas aeruginosa* resistance towards Ciprofloxacin (25%) was least compared to compared to Cephalosporin and Aminoglycosides. High resistance towards Meropenem (40%) was also recorded. More isolates from male participants were resistance to cephalosporins (CAZ 68%, FEP 53%) while those from females were more resistant to aminoglycoside (AK 80%, CN 41%). Bacteria isolates from participants aged between

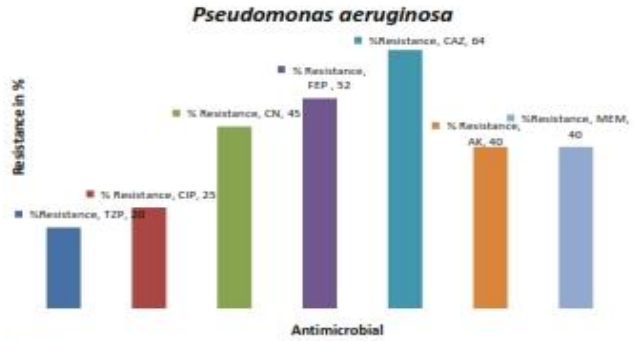

Figure 1. Antimicrobial resistance patterns of *Pseudomonas aeruginosa* isolates from wound samples. TZP: Tazobactum-piperacillin, CIP: Ciprofloxacin, CN: Gentamicin, FEP: Cefepime, CAZ: Ceftazidime, AK: Amikacin, MEM: Meropenem.

18 - 30 years were overall more resistant compared from those obtained from other age brackets (**Table 2**). A higher resistance towards virtually all tested antimicrobial agents was also recorded in bacterial isolates from participants who did not complete dosage compared to those that did.

3) Carriage of b-lactamase genes

bla_{NDM-1} was detected in 10 isolates from a collection of 34 isolates that were resistant to Meropenem. These isolates were resistant to virtually all cephalosporins and aminoglycosides. The isolates were also highly resistant to and also to Tazobactam-Piperacillin and Ciprofloxacin (60%). Carriage of TEM β-lactamase was the most predominant b-lactamase among the 34 isolates and in all isolates showing resistance to any b-lactam. Carriage of bla_{SHV} was also recorded amongst *Pseudomonas aeruginosa* isolates resistant to Ceftazidime.

4) Genetic relatedness of recovered *Pseudomonas aeruginosa* isolates

High genetic similarity based on (GTG) [5] fingerprint was noted among *Pseudomonas aeruginosa* isolates from diverse patients visiting Tigoni Hospital **Figure 2**. A similarity of ≥80% was also noted in 9 clusters based on isolates banding patterns, participant area of residence, and source of antimicrobial agents, resistance phenotype and genotype. Several *Pseudomonas aeruginosa* isolates with identical resistance phenotypes were clustered together (cluster 3). Despite of significant genetic similarity of the 9, most isolates had varying resistance phenotypes and genotype which perhaps is an indication that different clones have acquired these resistances independently. However the common factor among most isolates that clustered together was participant source of antimicrobial agent, either from private chemist or Hospital pharmacy. Notably the genetic basis of resistance (**Table 3**) in *Pseudomonas aeruginosa* isolates in the 9 cluster with significant genetic similarity was unknown, which suggest additional resistant mechanism from the targeted.

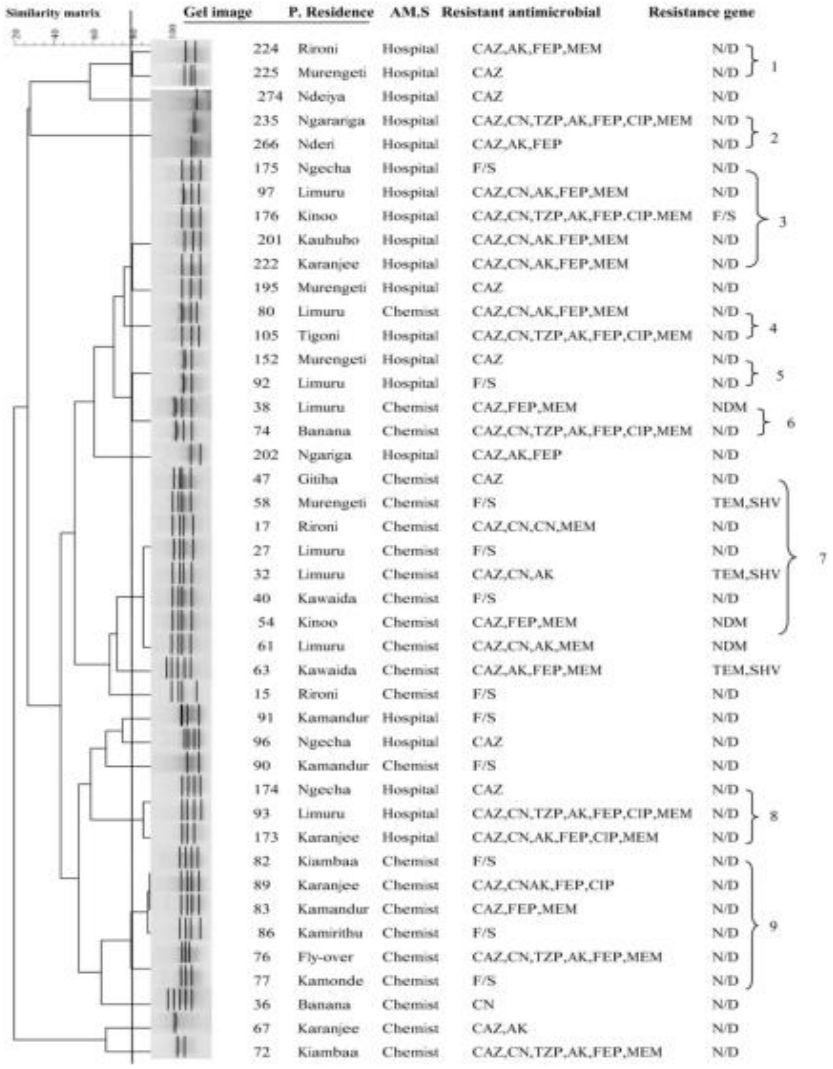

Figure 2. Fingerprint analysis of *Pseudomonas aeruginosa* isolates recovered from Patients visiting Tigoni Hospital. Key: P. residence: Participant's residence, AM.S: Source of antimicrobial, F/S: Fully susceptible, N/D: None of the target gene was detected.

Table 2. Antimicrobial resistance patterns of *P. aeruginosa* isolates in relation to demographic features of the study paricipants.

		Resistance %						
	n	**CN**	**TZP**	**CIP**	**FEP**	**CAZ**	**AK**	**MEM**
All	85	45	20	25	52	64	40	40
Gender of source								
Males	34	38	21	26	53	68	79	59
Females	51	41	20	24	51	67	80	73
Age of source								
Children < 13 years	5	20	0	0	60	80	100	80
Children 13 - 17	3	33	0	0	0	0	100	0
Adults 18 - 30	35	51	34	37	66	83	83	83
Adults 31 - 50	25	24	8	12	36	52	68	52
Adults > 50	17	47	18	29	53	71	82	65
Prescription adherance								
Dose completed	4	0	0	0	75	100	75	75
Dose incompleted	81	42	21	26	51	67	80	67
No prescription	66	44	23	24	47	65	79	64
with prescription	19	26	11	26	68	79	84	79
Source of drugs								
Drugs from hospitals	24	29	17	29	75	79	83	79
Drugs from chemist	61	44	21	23	43	64	79	62

TZP: Tazobactum-piperacillin, CIP: Ciprofloxacin, CN: Gentamicin, FEP: Cefepime, CAZ: Ceftazidime, AK: Amikacin, MEM: Meropenem, n: total count of corresponding feature.

Table 3. Resistant genes detected in *Pseudomonas aeruginosa* isolates.

Resistance gene detected	**Total detected**	**% AMR in *bla* positive isolates**						
		CAZ	**CN**	**TZP**	**FEP**	**CIP**	**MEM**	**AK**
bla_{TEM}	21	38	29	0	19	5	0	24
bla_{SHV}	20	40	30	0	15	5	0	5
bla_{NDM}	10	100	100	80	100	60	100	100

bla_{NDM}: New Delhi β-lactamase, bla_{SHV}: Sulhydryl β-lactamase variant, bla_{TEM}: Temoneria β-lactamase variant, AMR: Antimicrobial resitance.

5. Discussion

In the current study, we recovered 85 *Pseudomonas aeruginosa* from wound swabs obtained from 299 outpatients visiting Tigoni Hospital. Clinical isolates of *P. aeruginosa* have previously been documented in Kenya. Such studies include a cross-sectional study conducted in Aga Khan University Hospital. The study reported 416 *P. aeruginosa* urine, blood, wounds, purulent and respiratory tract specimens from patients admitted in Intensive care unit [6]. In this study, car-

riage of *Pseudomonas aeruginosa* in wound specimen was 30%, second to respiratory tract specimen (53%).

Our findings showed that adults are at a higher risk of carriage *Pseudomonas aeruginosa* in wounds infections compared to those of children with a significant statistical difference (P: 0.001, C.I: 2.1 - 14.0, O.R: 3.4). Similarly, female were more likely to be colonized at 67% carriage compared to those of males at 33% (P: 0.19, C.I: 0.84 - 2.4, O.R: 1.42). Further analysis also revealed that most individuals opted to obtain medication from community chemists as opposed to hospital pharmacies with a significant difference in carriage of *P. aeruginosa* in wound infections been noted (P: 0.001, C.I: 3.01 - 8.86, O.R: 5.17). Although we did not go a further notch to determine the possible cause of the above difference, it is very likely that some of the medicine available in the community chemist are counterfeits therefore not effective in clearing infections. Self-medication is a major public health problem and possible driver of antimicrobial resistance in the country. Miss-use of antimicrobial agents was not an exception among study participants where the menace was significantly associated with *Pseudomonas aeruginosa* carriage (P: 0.001, C.I: 3.01 - 8.86, O.R: 5.17). It was also noted that most of the self-medicating did not have disease diagnosis and therefore medication was non-specific and often not in full dosage. It was further noted that majority of self-medicating individuals did not have a doctor's prescription. Poverty and high doctor's consultation fee were noted as major cause of self-medication preference among this population.

In addition to the intrinsic antimicrobial resistance in *Pseudomonas aeruginosa*, misuse and over use of antimicrobial agents have led to emergence of multiple resistance strains that are difficult to treat. In addition, *P. aeruginosa* have become resistance to antimicrobial agents through horizontal acquisition of resistance genes from resistant strains and other bacterial species such *Acinetobacter baumannii*. Findings of the current study indicate an overall resistance to test antimicrobial agents compared to a study conducted in Kenyatta National hospital that recorded high resistance to CAZ 70%, AK 46%, CN 67.9%, CIP 52.7%, TZP 50.5% and MEM 67.6% [13] In this study were recorded high resistance prevalence to important classes of antibiotics such as CAZ (64%), Gentamicin at 45% and Ciprofloxacin at, 25%. A combination of these antimicrobials is used in the treatment of severe *P. aeruginosa* infections and therefore, resistance to a combination of such agents may greatly hamper treatment. Resistance to carbapenem which is the last resort was also recorded (MEM, 40%). Our findings therefore show an increase in carbapenem resistance in East Africa as compared to a systematic review done in the region that reported an incidence of 21% [14]. Tazobactam-Piperacillin the most used anti-Pseudomonal agent was effective to most isolates that were resistant to other antimicrobials

Resistance to third generation β-lactams such as CAZ has been attributed to carriage of β-lactamases [15] bla_{NDM} carriage was positive in only 10 from a total of 34 isolates that were resistant to Meropenem. However, only 40% of these

isolates with this gene were positive for bla_{TEM} or bla_{SHV} indicating that there is little evidence of co-carriage of this gene with other bla genes as described in a recent study on *P. aeruginosa* strains from Kenyatta hospital wards in Kenya [13]. The above mentioned study also recorded 51.9% bla_{NDM} and 49.6%. bla_{VEB} carriage among 188 *Pseudomonas aeruginosa* isolates from urine, blood, respiratory tract and pus specimen. This may therefore infer possible carriage of other β-lactamase genes in our *Pseudomonas aeruginosa* isolates [3]. However, the DNA extracts of these bacterial isolates have since been sent for whole genome sequencing which further help in determine their genetic structure. Some meropenem isolates tested negative for the other carbapenems and these have been submitted WGS. We recorded high resistances to β-lactams including carbapenems. It is significant that these isolates were obtained from people who were not necessarily treating wounds. Since some of the wounds were open, there is a possibility that such strains may be coming from the environment especially soil.

The phylogeny of recovered *strains* revealed significant genetic similarity among our bacterial isolates. Further analysis also showed possible clonal expansion of resistant strains within the hospital that has identical resistance phenotype and a high genetic similarity. Tight clustering of isolates with different resistance phenotypes further suggests independent acquisition of a similar set of resistance determinants among isolates with different profiles. Although we were not able to determine possible carriage of genetic elements such as plasmids that could possible cause above mention resistance features, further genetic analysis and SNP typing based on WGS data will shed light into this. Although (GTG)5 is not a very powerful phylogeny method, there is possibility that the NDM strains are clonal going by their clustering and resistance profiles. It is worth noting that the non-NDM strains did not cluster tightly which raises the possibility of clone stability of the highly resistant NDM strains.

The spread of clonal strains in clinical set-up has been associated with use medical devices such as catheters and prosthetics [16]. *Pseudomonas aeruginosa* strains are able to survive in the environment especially in most areas. Therefore, person to person transmission is also possible especially in contaminated environment. There should therefore be deliberate effort to reduce transmission and source reservoir of such resistance strains in such settings.

6. Conclusion

The emergence of bla_{NDM-1} linked to widespread misuse of carbapenem is alarming. Measures to institutionalize antimicrobial stewardship such as hospital committees, prescription guided by empirical laboratory susceptibility patterns and continued monitoring of development of resistance should therefore be initiated. Risk factor associated with carriage and acquisition of multiple-drug resistance strains such as self-medication should therefore be strongly discouraged. Measures to closely monitor dosage completion and post-treatment follow-up for serious infections should also be initiated where possible to prevent

emergence of antimicrobial resistance.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Appendix VII: KEMRI **Ethical Approval Letter**

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 December 16, 2014

TO: THUO THOMAS GACHUKI,
PRINCIPAL INVESTIGATOR

THROUGH: DR. WILLIE SANG,
THE DIRECTOR, CMR,
NAIROBI

RE: SSC PROTOCOL NO. 2911 (*RESUBMISSION*): CROSS SECTION STUDY ON SUSCEPTIBALITY PATTERNS AND PRESENCE OF AAC-(6') –IB-CR GENES IN PSEUDOMONAS AERUGINOSA INFECTIONS IN A RURAL HOSPITAL IN KENYA (*VERSION 25/11/2014*)

Reference is made to your undated letter of which the Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study protocol on December 2, 2014.

This is to inform you that the Ethics Review Committee (ERC) reviewed the documents submitted and is satisfied that the issues raised at the 232nd meeting of the KEMRI ERC on 21st October, 2014 have been adequately addressed.

The study is granted approval for implementation effective this **16th December, 2014**. Please note that authorization to conduct this study will automatically expire on **15th December, 2015.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to SERU by **November 4, 2015**.

Any unanticipated problems resulting from the implementation of this protocol should be brought to the attention of the SERU. You are also required to submit any proposed changes to this protocol to SERU prior to initiation and advise SERU when the study is completed or discontinued.

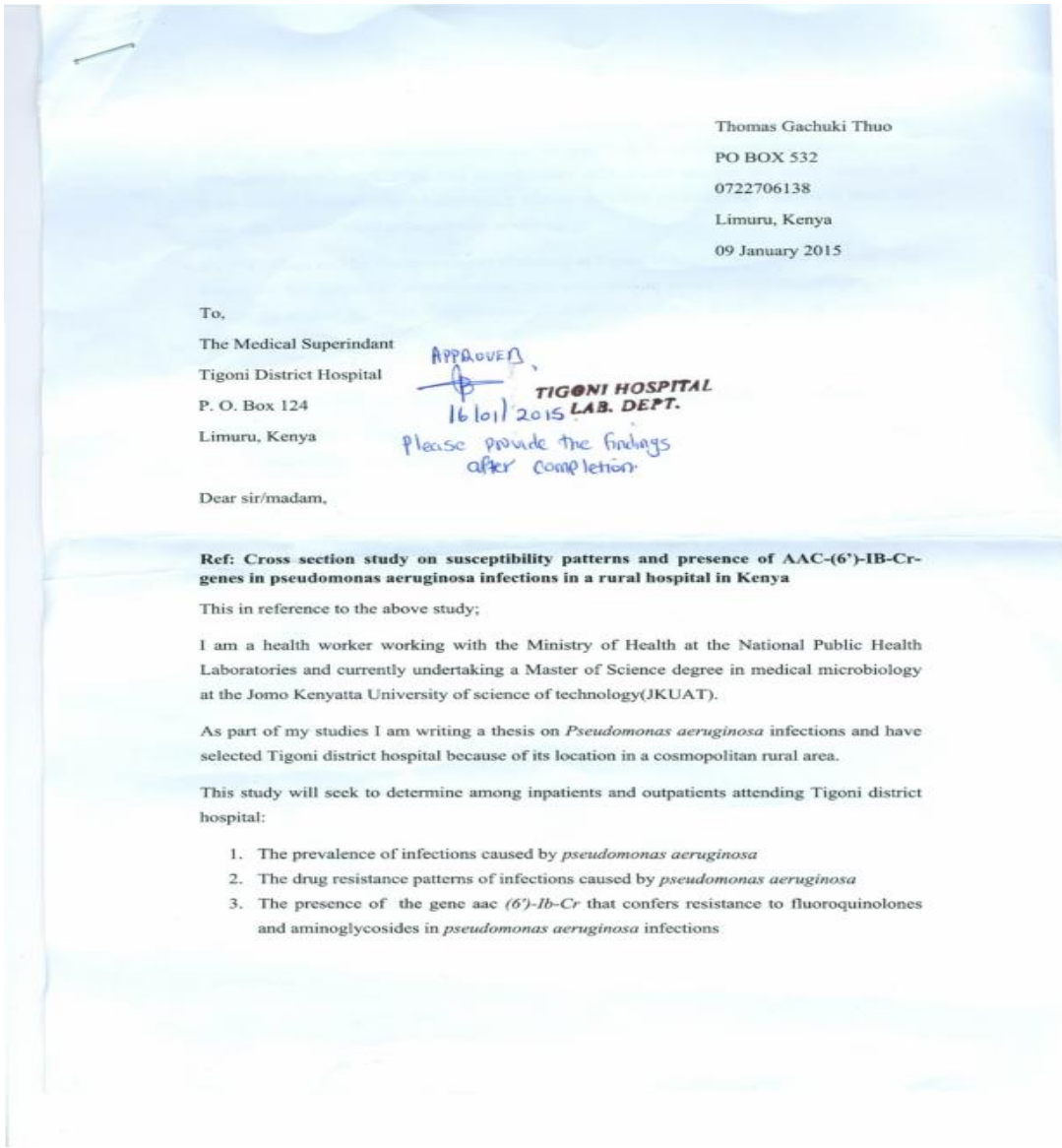
You may embark on the study.

Yours faithfully,

PROF. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI/ETHICS REVIEW COMMITTEE

In Search of Better Health

Appendix VIII: Letter of Approval Tigoni



Thomas Gachuki Thuo
PO BOX 532
0722706138
Limuru, Kenya
09 January 2015

To,
The Medical Superindant
Tigoni District Hospital
P. O. Box 124
Limuru, Kenya

APPROVED
16/01/2015
TIGONI HOSPITAL
LAB. DEPT.
Please provide the findings after completion.

Dear sir/madam,

Ref: Cross section study on susceptibility patterns and presence of AAC-(6')-IB-Cr-genes in pseudomonas aeruginosa infections in a rural hospital in Kenya

This in reference to the above study;

I am a health worker working with the Ministry of Health at the National Public Health Laboratories and currently undertaking a Master of Science degree in medical microbiology at the Jomo Kenyatta University of science of technology(JKUAT).

As part of my studies I am writing a thesis on *Pseudomonas aeruginosa* infections and have selected Tigoni district hospital because of its location in a cosmopolitan rural area.

This study will seek to determine among inpatients and outpatients attending Tigoni district hospital:

1. The prevalence of infections caused by *pseudomonas aeruginosa*
2. The drug resistance patterns of infections caused by *pseudomonas aeruginosa*
3. The presence of the gene aac *(6')-Ib-Cr* that confers resistance to fluoroquinolones and aminoglycosides in *pseudomonas aeruginosa* infections

The findings are therefore expected to inform clinicians on the various susceptibility patterns of drugs and provide vital information on the organism for planners in the country. Study participants will benefit from free microbiology culture services that are study related and from reliable treatment by administering locally available antimicrobials shown to work in-vitro after antimicrobial susceptibility profiling.

Study findings will be disseminated to clinicians at Tigoni District Hospital in a planned open forum by the hospitals' administration, and presented in antimicrobial resistance forums locally to enable informed formulations of antimicrobial use guidelines and policies in Kenya.

Consent will be obtained from all participants who will attest to this by signing consent forms. Confidentiality will be safe guarded at all times. The study targets **113 respondents**.

Consent to conduct the study has been obtained from the KEMRI scientific and ethical review board (ERC) for a period of one year. Study commences immediately subject to your approval

Kindly find attached copies of the ERC approval, study protocol, consent forms and study questionnaire. If you require additional information I am readily available.

Thank you and hoping for a favourable response

Thomas Gachuki Thuo